EXPERIMENTAL AMYLOIDOSIS

Studies With a Modified Casein Method, Casein Hydrolysate and Gelatin

David T. Janigan, M.D.

From the Department of Pathology and Oncology, University of Kansas Medical Center, Kansas City, Kansas

Numerous experimental procedures have been used to induce amyloidosis in various animal species. Many investigators using casein injections, the most popular method, have commented on the variations in onset of amyloidosis, even within one inbred strain.[1] The present paper reports (1) a modified casein injection method which regularly produced amyloidosis of fairly uniform onset in mice and (2) the results of a study comparing the amyloid-provoking capacity of three protein solutions of varying antigenicity.

Material and Methods

Male C57BL/10J mice, 6 to 7 weeks old, from the Jackson Memorial Laboratory, Bar Harbor, Maine, were used. Random necropsies prior to the experiments revealed no evidence of infection. The mice were housed in groups of 5 or 6 in plastic cages in an air-conditioned room, maintained between 70 to 75° F. The diet consisted of Purina® Laboratory Rat Chow (crude protein content not less than 23 per cent) and tap water.

Protein Solutions

(*a*) *Casein.* Five gm of casein, Hammersten grade, Lot No. 7006, (Nutritional Biochemicals Corp., Cleveland, Ohio) was dissolved slowly, over 5 to 6 hours, by sprinkling into 100 ml 0.0075 M sodium bicarbonate in a wide-bottom beaker at 50 to 60° C. Fluid lost by evaporation, usually amounting to no more than 15 ml, was replaced throughout the preparation by appropriate amounts of bicarbonate. Some clumps of casein remained undissolved and were removed by filtration through gauze cloth; the amount was generally no greater than 200 to 300 mg. After cooling, the casein solution was enclosed in dialysis tubing, placed over a large fan and evaporated to a volume between 50 to 60 ml, yielding a final casein concentration between 7 to 10 gm per cent, as measured by the biuret reaction. The final estimated molarity of sodium bicarbonate was about 0.020 and the pH, with litmus paper, 6 to 6.5. Casein solutions became to viscous if concentrated beyond 10 gm per cent and subcutaneous fibrosis was stimulated, on injection, if the estimated molarity of bicarbonate approached or exceeded 0.030.

(*b*) *Casein Hydrolysate.* An enzymatic hydrolysate of casein (Nutritional Biochemical Corp., Cleveland, Ohio) was dissolved slowly in 0.015 M sodium bicarbonate to a final concentration of 8 gm per cent with gentle heating. No trichloroacetic acid-precipitable material was detectable in these preparations.

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(*c*) *Gelatin.* Both an 8 and 16 gm per cent solution of gelatin (Difco Laboratories, Detroit, Mich.) was prepared as in (b).

All solutions were kept at 2 to 4° C and were not sterilized. They were injected either subcutaneously or intraperitoneally in 0.3 ml quantities according to the schedules given in Tables I and II. Control animals were given injections of 0.3 ml of 0.015 M sodium bicarbonate. Subcutaneous injection sites used in daily rotation were neck, back and thighs.

The mice were killed by cervical dislocation 24 hours after the last injection. At necropsy, livers and spleens were weighed. Small blocks of all organs, except brain and bone, were fixed for 24 hours in cold 4 per cent formaldehyde containing 0.22 M sucrose buffered to pH 7.2 [2] and embedded in paraffin after washing overnight

TABLE I

HEPATIC AND SPLENIC AMYLOIDOSIS *

Material injected §	Amount injected (mgm)	Day killed	Weight gain (gm)	Organ weights † (% body weight)		Incidence of amyloidosis	
				Liver	Spleen	Liver	Spleen
No injection (5)	—	62	8.0 ± 1.4	5.1 ± 0.31	0.31 ± 0.017	—	—
			Subcutaneous injections				
$NaHCO_3$ (5)	—	14	1.8 ± 0.27	5.7 ± 0.48	0.27 ± 0.025	—	—
" (7)	—	21-23	1.3 ± 0.83	5.2 ± 0.33	0.33 ± 0.063	—	—
" (4)	—	28	3.4 ± 0.26	5.5 ± 0.2	0.31 ± 0.055	—	—
" (9)	—	35-41	3.5 ± 0.7	5.3 ± 0.16	0.30 ± 0.06	—	—
" (4)	—	51	3.1 ± 0.62	5.5 ± 0.22	0.30 ± 0.017	—	—
" (8)	—	70	4.2 ± 0.85	5.7 ± 0.64	0.34 ± 0.024	—	—
Casein (5)	238	14	1.1 ± 0.66	5.9 ± 0.34	0.37 ± 0.077	—	—
" (11)	337-388	21-23	1.8 ± 0.7	5.5 ± 0.3	0.42 ± 0.08	—	—
" (6)	460	28	2.3 ± 0.59	6.2 ± 0.27	0.39 ± 0.06	—	4/6
" (8)	520	31	—	—	—	6/8	8/8
" (14)	600-675	35-41	2.7 ± 1.34	6.0 ± 0.23	0.54 ± 0.077	12/14	14/14
" (6)	862	51	2.7 ± 1.34	7.3 ± 0.94	0.64 ± 0.052	6/6	6/6
" (6)	1151	70	1.6 ± 0.87	8.0 ± 0.46	0.71 ± 0.077	6/6	6/6
Casein Hydrolysate (5)	600	35	2.5 ± 0.5	5.6 ± 0.05	0.35 ± 0.083	—	—
" (5)	720	41	4.9 ± 1.58	5.3 ± 0.09	0.44 ± 0.087	—	—
" (5)	1320	62	6.6 ± 1.76	5.3 ± 0.31	0.35 ± 0.028	—	—
Gelatin (5)	600	35	3.1 ± 0.78	5.4 ± 0.27	0.30 ± 0.054	—	—
" (5)	720	41	4.3 ± 1.26	5.6 ± 0.17	0.37 ± 0.035	—	—
" (5)	1320	62	4.7 ± 0.91	5.5 ± 0.22	0.41 ± 0.041	—	—
" (4)	1800	35	5.0 ± 0.81	5.6 ± 0.42	0.45 ± 0.067	—	—
			Intraperitoneal injections				
$NaHCO_3$ (4)	—	35-41	3.2 ± 0.36	5.2 ± 0.2	0.32 ± 0.04	—	—
" (4)	—	62	4.4 ± 1.7	5.6 ± 0.51	0.41 ± 0.052	—	—
Casein (5)	600	35	1.1 ± 1.08	6.1 ± 0.44	0.4 ± 0.1	4/5	5/5
" (4)	750	41	2.9 ± 0.63	5.8 ± 0.4	0.44 ± 0.07	4/4	4/4
Casein Hydrolysate (5)	1320	62	5.2 ± 2.5	5.5 ± 0.24	0.36 ± 0.023	—	—
Gelatin (5)	1320	62	5.3 ± 1.14	5.4 ± 0.22	0.33 ± 0.017	—	—

* Mice given daily injections (casein, casein hydrolysate and gelatin) 5 days a week for varying periods of time. All mice killed 24 hours after last injection.

† All weight values represent means with standard deviations.

§ Number of animals in parenthesis.

in cold distilled water. Sections were cut at 2 to 4 μ and stained with the periodic acid-cold-Schiff (PAS) reagent.[3] The following staining criteria were used to identify amyloid in the first 70 mice and thereafter when required: (1) Congo red binding, with positive birefringence and dichroism of the amyloid-Congo red complex in

TABLE II

INCIDENCE OF HEPATIC AND SPLENIC AMYLOIDOSIS *

Number of mice	Amount injected (mgm)	Day killed	Incidence of amyloidosis	
			Liver	Spleen
5	500	22	4/5	5/5
5	648	29	5/5	5/5

* Mice given daily injections of casein 7 consecutive days a week for 3 and 4 weeks, and killed 24 hours after the last injection.

polarized light [4]; (2) metachromasia after crystal violet [4]; (3) fluorescence in ultraviolet light after staining with thioflavine T [5]; (4) positive indole reaction for tryptophane.[6]

The severity of hepatic and splenic amyloidosis was estimated subjectively as follows: "slight" when amyloid was just recognizable in perifollicular zones of the spleen (Fig. 1), and in portal veins (Fig. 4) and peripheral sinusoids of the liver, and requiring the above staining procedures for confirmation; "moderate" when easily recognizable (Figs. 2 and 5), and "extensive" when compression of adjacent structure by amyloid was obvious (Figs. 3 and 6).

RESULTS

With any one group of mice developing amyloidosis, the degree of involvement of either the spleens or of the livers was fairly uniform. The histologic features of the deposits were similar to those described previously [7-9]; at all times the deposits stained with Congo red and thioflavine T, showed crystal violet metachromasia and gave a positive indole reaction.

Subcutaneous Injections

Onset of Amyloidosis. Whereas the spleen showed only slight degrees of amyloidosis in 4 of 6 mice after injection of 460 mgm casein, all mice receiving 520 mgm developed slight to moderate splenic amyloidosis (Table I). At the latter dosage level, 75 per cent developed slight hepatic amyloidosis. With continued casein injections the incidence (Table I) and severity of amyloidosis in both organs increased.

When a total of 500 and 648 mgm of casein was given over a 22- and 29-day period respectively (Table II) the incidence of hepatic and splenic amyloidosis was almost identical to that found when similar amounts were injected over 31 to 41 days (Table I). The severity of amyloidosis was, however, less uniform in the group treated for the shorter interval.

Casein hydrolysate and gelatin, in large quantities (Table I), as well as sodium bicarbonate, failed to provoke amyloidosis. No spontaneous amyloidosis was observed in mice not receiving injections.

Histologic Features. As noted above, the morphologic appearance and localization of amyloid in the spleen and liver were similar to those described by others. It was notable, however, that no plasma cell or histiocyte infiltrates were found to precede or accompany early hepatic amyloid deposits; in the spleen this feature was difficult to evaluate. In contrast, during the later stages of amyloid formation, i.e. after injection of 600 or more mgm casein, numerous aggregates of plasma cells and histiocytes were frequently observed around amyloid deposits in both organs. These cells were occasionally arranged in a corona-like pattern around the periphery of the deposits (Fig. 6).

Changes at Injection Sites. Subcutaneous reactions to the first 21 to 30 injections of casein were usually slight, being similar to those found after casein hydrolysate or gelatin. The chief difference was the presence of small numbers of plasma cells in the casein groups. After 36 to 50 casein injections, in excess of the number found to induce amyloidosis, slight to moderate degrees of subcutaneous fibrosis developed with increasing numbers of lymphocytes, plasma cells and reactive giant cells. No ulcers or subcutaneous amyloid developed in any of the mice.

Organ Weight Changes. Splenic and hepatic weights, both absolute and relative to body weight, increased after casein injections (Table I); before the accumulation of extensive deposits of amyloid, this increase was due in part to hypertrophy and probably hyperplasia of sinusoidal cells in the liver, and to reticulum cell and lymphoid hyperplasia in the spleen. Axillary and inguinal lymph nodes draining the injection sites were markedly enlarged and also showed lymphoid and reticulum cell hyperplasia with focal aggregates of plasma cells, but no amyloid. In contrast, similar changes were much less marked in mice given casein hydrolysate and gelatin but, in turn, were slightly greater than in mice which were either untreated or injected with sodium bicarbonate (Table I). The mice receiving casein injections showed the lowest over-all body weight gain, contributing in part to the higher liver and spleen: body weight ratios found in this group (Table I).

Intraperitoneal Injections

Onset of Amyloidosis. The time of onset and the incidence of splenic and hepatic amyloidosis induced by intraperitoneal casein were similar to that found after subcutaneous injection of similar quantities (Table I). The severity of amyloidosis, although somewhat less uniform than in the latter group, did not vary markedly.

Intraperitoneal injections of casein hydrolysate, gelatin and sodium bicarbonate failed to induce amyloidosis (Table I).

The cellular reactions associated with amyloid deposits and the organ weight changes in response to injections of the 3 solutions were similar to those recorded after subcutaneous injections (Table I).

Peritoneal Reactions. After casein injections, mild peritoneal edema was observed with cytoplasmic swelling and vesiculation of mesothelial cells. In almost all mice, the liver capsules were slightly opaque, due to fibrous thickening, but were free from amyloidosis. Enlarged mesenteric lymph nodes showed reticulum cell and lymphoid hyperplasia with plasma cells, but no amyloid. The peritoneal reactions after casein hydrolysate, gelatin and sodium bicarbonate injections were qualitatively similar but less marked.

Discussion

This report describes a casein injection method for the regular production of amyloidosis in mice. The results contrast with the experience of Kennedy who stated that the occurrence of amyloidosis was "fickle, unpredictable, and unrelated to strain, sex, age or material injected," and who cited similar results reported by others.[1]

As a result of the present studies we feel that such variability may be related, in part, to the properties of the casein solutions used for injections. Because of its limited solubility, casein is customarily dissolved in relatively strong alkaline solutions, i.e. 0.06 M or 0.1 M. sodium hydroxide,[10] to a final concentration of 5 gm per cent. The pH of these solutions ranges from 8 to 10 and considerable daily volumes, 0.5 to 1.0 ml, are injected subcutaneously in mice for periods up to 3 to 4 months.[1,11] There are infrequent comments on the tissue changes at the injection sites of these preparations.[12–14] In pilot experiments which preceded the present study, however, extensive subcutaneous fibrosis and frequent skin ulcers were found when similar casein solutions, even sterilized, were injected into mice of three separate strains. While amyloidosis was produced, the onset and severity did indeed vary markedly and appeared to correlate best with the severity of tissue reactions at the injection sites. Similar reactions may have prompted one group to refer to repeated casein injections as one of the "chronic inflammatory stimuli" used to induce amyloidosis.[15]

The method reported here yields casein solutions of high protein and low ionic base concentration. With these properties, daily injections of a relatively small volume provoke neither severe fibrosis nor ulcerations at injection sites. In the absence of such complications, the onset of

amyloidosis could be predicted with reasonable uniformity on the basis of the quantity of casein injected.

Since non-sterilized casein solutions were used, the role of bacterial contamination cannot be excluded completely. The following points are offered, however, to suggest that such a role, if operative, was of minor importance: gross and histologic absence of abscesses or bacteria; early and uniform onset of amyloidosis in contrast to the later and variable onset when amyloidosis was provoked by bacterial infections [16]; absence of amyloid in mice given injections of casein hydrolysate and gelatin, solutions prepared and administered under the same condition, and therefore equally susceptible to bacterial contamination.

Accumulations of plasma cells, histiocytes or increases in fixed reticuloendothelial cells have been observed to precede or accompany the formation of experimental amyloidosis in the liver or spleen.[1,9,13,17] The intimate association of these cells with extracellular amyloid fibrils has been emphasized in recent electron microscopic studies,[11,18,19] and some have suggested that the extrusion and extracellular precipitation of the cytoplasmic contents of plasma cells or histiocytes is the source or precursor of amyloid.[11,19] Without refuting this possibility, it is notable that some of these observations were based on tissues obtained from prolonged experiments,[11,13,17] e.g., daily casein injections in mice for 3 months, resulting in extensive splenic amyloid.[11] Once deposited, amyloid can provoke histiocytic and giant cell, as well as plasma cell, infiltrations, particularly if affected animals are allowed to live for long periods.[14,20,21] Similar infiltrates were observed in the present studies if casein injections were maintained over 5 to 10 weeks, well after the initial onset of amyloid. In accord with Heefner and Sorenson,[22] however, no plasma cells or histiocyte infiltrates were observed during the early phases of amyloid formation. Therefore, in studies on the cellular histogenesis of amyloid, it may be advantageous and more informative to concentrate upon the earlier stages of formation which can be predicted reasonably by the present method.

Role of Immune Mechanisms in Amyloidosis

Immunologic responses to antigenic stimulation are considered by many to be a prime pathogenetic mechanism involved in experimental and in some forms of human amyloidosis.[21] A review of the former shows that amyloidosis has been induced in various species by injections of bacteria and protozoa,[1,16,23] bacterial toxins,[9,16,24] Freund's adjuvant,[1,25,26] egg albumin,[27,28] ribonucleic acids [12,20,27,29,30] gelatin and zein,[27] peptones,[27,31] sterile pancreatic extracts (? inactivated),[32] blood proteins,[23] turpentine,[16] pancreatin and papain,[16] colloidal sulfur,[27]

phenolic solutions,[33] saline[33–35] and distilled water.[33] Amyloidosis has also been induced by dietary factors, including protein[36–38] or cholesterol excess,[39] vitamin C deficiency,[40] and goitrogens,[41] and by gamma radiation,[42] tumor transplantation,[10,29] parabiosis,[43–45] ligation of blood supply to various organs,[43] and by electroshock stress of mice after unilateral nephrectomy.[46] In many of these reports other amyloid-provoking procedures are cited, e.g., injections of sodium hydroxide, sodium bicarbonate, hydrochloric acid, alloxan, animal tars and injections or feeding of silicates. While some of these procedures clearly constitute some form of antigenic challenge in experimental animals, the mechanisms involved in others are not readily apparent.

In addition, the results of studies designed to test the effect of altered immunity on experimental amyloidosis are conflicting: different investigators have observed that the development of casein-induced amyloidosis was either prevented, accelerated or affected little by either splenectomy, cortisone, adrenocorticotrophin, nitrogen mustard or irradiation.[1,8–10,47–53] Similarly, the reported effects of diet and of other procedures on casein-induced amyloidosis are also conflicting or difficult to interpret: (1) findings of prevention or retardation of amyloidosis in mice on a high protein,[54] high cholesterol[54] or normal diet[47,55] contrast with the report of a higher incidence in mice on a normal or high protein diet than in those on a protein-poor diet;[34] (2) enhancement by either scorbutigenic diets[35,47,48] or by ascorbic acid injections (see page 129, reference 8); by thyroidectomy and castration,[48] thyrotrophin injections[35] or increasing body temperature.[47]

Finally, an analysis of the results observed in many of these experimental studies is further complicated by uncertainty concerning the influence of concomitant bacterial infections,[16] the criteria used to identify amyloid,[45,56,57] and spontaneous amyloidosis.[58] The recent characterization of amyloidosis in ducks as a spontaneous occurrence[59] rather than the result of methylcholanthrene applications, as previously thought,[60] attests to the importance of these considerations.

Although the factors involved in amyloid formation remain to be elucidated, the results of the present study indicate clearly that adequate antigenic stimulation of mice appears to be at least one requisite. Gelatin, although once erroneously considered to be a non-antigenic protein is, nevertheless, very weakly antigenic[61] and casein hydrolysate is non-antigenic. Injections of both failed to provoke amyloid. In other unpublished studies,[62] however, amyloidosis was induced in mice by injections of gelatin only after the latter was coupled to a diazonium compound, a procedure which confers greater antigenicity upon this protein.[63]

Summary

A modified casein method for the production of amyloidosis with a predictable and fairly uniformly early onset in mice is described. The advantage of studying the early stages of amyloid formation and the reasons allotted thereto are emphasized. Failure to produce amyloidosis with weakly or non-antigenic protein substances was observed. These results are discussed in terms of the necessity to recognize certain simultaneous variables, biologic as well as technical, in experimental models of amyloid induction.

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Addendum

Since submitting this manuscript, tests on an improved casein method were completed. The method permits rapid solubilization of casein and earlier amyloid induction: dissolve 10 gm casein in 200 ml of 0.05 M $NaHCO_3$ and dialyze against cold distilled water for 48 hours. Following dialysis, evaporate to 100 ml to obtain a 10 gm per cent casein solution with a $NaHCO_3$ molarity of less than 0.010.

[*Illustrations follow*]

Legends for Figures

All sections are from formaldehyde-fixed tissues stained by Lillie's periodic acid-cold-Schiff technique.

Fig. 1. Mouse received 460 mgm casein. Slight amyloidosis appears between cells (arrows) at the periphery of a splenic follicle; germinal center is at lower right and a fibrous trabecula at the extreme upper left. × 250.

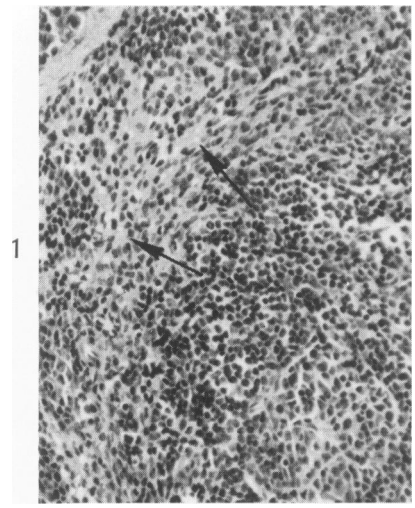
Fig. 2. Mouse given 520 mgm casein. Moderate amyloidosis is evident around a splenic follicle. × 100.

Fig. 3. Mouse given 862 mgm casein. Extensive splenic amyloidosis is associated with compression and obliteration of follicular pattern. × 100.

Fig. 4. Mouse given 520 mgm casein. Slight hepatic amyloidosis involves the subendothelial layers of a small portal vessel. × 820.

Fig. 5. Mouse given 675 mgm casein. There is moderate hepatic amyloidosis of portal vein tributaries and sinusoids. × 140.

Fig. 6. Mouse given 862 mgm casein. A very pale staining amyloid deposit is surrounded by numerous reactive cells, many of which are histiocytes; the cytoplasm of many of these cells merges imperceptibly with the amyloid material and contains PAS-positive granules. × 820.



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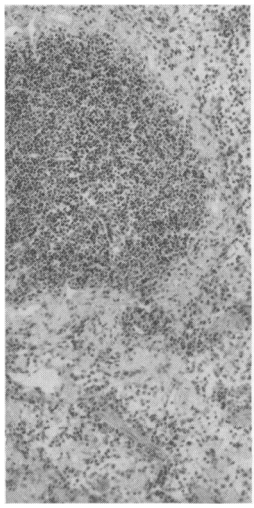

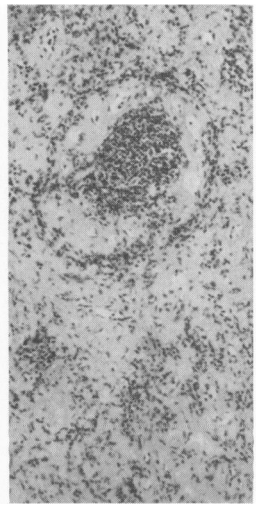

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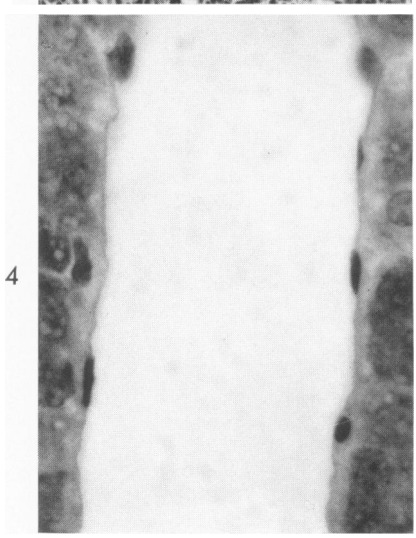

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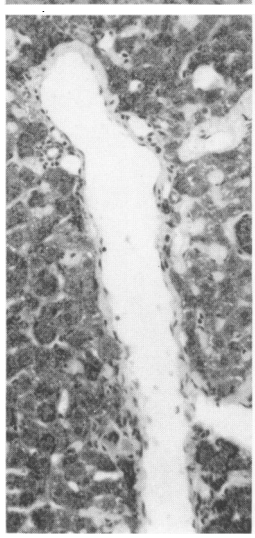

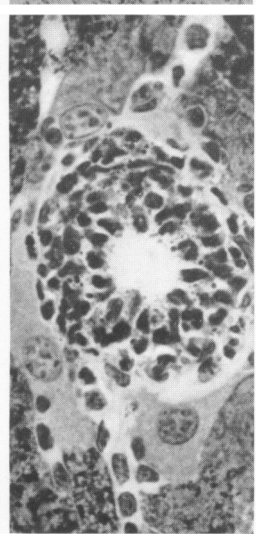

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